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The Effect of Temperature on the Binding of Sulfonamides to Carbonic Anhydrase Isoenzymes I, II, and IV

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SUMMARY

We report the effect of temperature on the equilibrium dissociation constants (K_I) for a series of six sulfonamides binding to three carbonic anhydrase (CA) isoenzymes (I, II, and IV). K_I values obtained at 0°, 15°, and 23° under conditions of nearly constant and low substrate (CO_2) concentration were used to calculate enthalpy and entropy changes associated with sulfonamide binding as well as to provide estimates of inhibitory potency of sulfonamides at 37°. We studies four classic sulfonamides (methazolamide, benzolamide, ethoxzolamide, and sulfanilamide) and the novel sulfonamides MK-507 (dorzolamide) and $CF_3SO_2NH_2$. In all cases, the K_I was observed to increase with increasing temperature, which is consistent with a negative enthalpy of sulfonamide binding. The extrapolated increase in K_I over the 0–37° temperature range varied from 4-fold for sulfanilamide binding to CA I to 14-fold for $CF_3SO_2NH_2$ binding to CA IV, corresponding to binding enthalpy values of −7.2 to −11.7 kcal/mol. For CA II and I, entropy changes associated with sulfonamide binding were in general modest and ranged from −5.3 to +4.1 entropy units (eu) for five of the compounds tested. In contrast, ethoxzolamide binding was associated with a relatively large positive entropy change. Also, the variatione in k_{on} and k_{off} with temperature were studied for three sulfonamides binding to CA II. The association rate constants for methazolamide, benzolamide, and ethoxzolamide binding showed increases of 2-fold or less, whereas dissociation constants increased 3–9-fold over the range of 0–37°. Thus, the temperature effect in increasing K_I is in large part due to a faster rate of sulfonamide dissociation. Apparent activation parameters at 23° for k_{on} were $\Delta H‡ = -2.35$ to 3.8 kcal/mol, $\Delta G‡ = 7.3$ to 8.6 kcal/mol, and $\Delta S‡ = -16.2$ to −32.7 entropy units. For k_{off}, the corresponding values were $\Delta H‡ = 5.6$ to 14.5 kcal/mol, $\Delta G‡ = 19.0$ kcal/mol, and $\Delta S‡ = -14.8$ to −45.7 entropy units.

The unsubstituted aromatic and heteroaromatic sulfonamides represent a very large class of specific and potent inhibitors of the CAs, which are zinc metalloenzymes of molecular mass 30–56 kDa. There are seven recognized isoenzymes of this family; they may broadly be classified according to the efficiency with which they catalyze the interconversion of CO_2 and HCO_3^-. At one extreme are the high activity isoenzymes CA II (cytosolic) and CA IV (membrane bound), which in concert or individually have roles in fluid and ion transport in secretory tissues and have established roles in the treatment of several diseases, notably, glaucoma and altitude sickness (1, 2). These may be contrasted with lower activity isoenzymes typified by CA I (e.g., erythrocyte, gut) and CA III (e.g., muscle, adipose tissue), which are approximately 3 and 800 times less efficient in CO_2 hydration, as determined by the respective k_{cat}/K_m values at 0° (3, 4); also, precise physiological roles for these isoenzymes have not yet been found.

The interaction of sulfonamides with CA isoenzymes is believed to be a two-step process involving a loose association of neutral (un-ionized) sulfonamides with the enzyme followed by a rate-limiting step in which sulfonamide nitrogen bonds to enzyme-ligated zinc (5). Inhibition by sulfonamides of CA catalysis is a direct result of displacement of zinc-bound hydroxyl ion (E-Zn-OH^-) by sulfonamide anion RSO_2NH^-, resulting in formation of the enzyme-inhibitor complex (E-Zn^-NHSO_2R). E-$ZnOH^-$ is the nucleophilic species that hydroxylates CO_2 in the catalytic pathway, producing HCO_3^- (6–9). Two ionizations, one activity linked (E-Zn-H_2O ⇄ E-Zn-OH^-) and the other related to sulfonamide ionization (RSO_2NH_2 ⇄ RSO_2NH^-), are found to have an influence on the association rate constant (k_{on}) and indirectly on K_I ($= k_{off}/k_{on}$). Protonation of the enzyme-linked ionization or deprotonation of the sulfonamide leads to a 10-fold reduction in k_{on}, but at pH 7–9, k_{on} is essentially unchanged, as is k_{off} over a wide range of pH (10).

There are no previous reports on the effect of temperature on k_{on} or k_{off} for any of the CAs. In a previous study with CA II, we demonstrated a weakening of sulfonamide inhibition (increase in K_I) by factors of 2–60-fold when temperature was raised from 0° to 37° (11). Two articles have reported enthalpy and entropy values for sulfonamide binding, but these did not include direct data on the effect of temperature on K_I (12, 13). In both studies, the observed entropy changes asso-

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ABBREVIATIONS: CA, carbonic anhydrase; EU, enzyme units.

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ciated with sulfonamide binding were small, leading to the conclusion that inhibitor binding to CA is an enthalpy-driven reaction opposed by small or moderate entropy changes.

In the present study, the binding of aromatic and heteroaromatic sulfonamides of diverse physicochemical type to three CA isoenzymes (CA I, II, and IV) has been investigated over the 0–23° temperature range. We also studied the aliphatic sulfonamide $CF_3SO_2NH_2$, an atypical CA inhibitor (14) that has great inhibitory potency yet lacks hydrophobic ring structure. Estimates are provided for the inhibitor potency of sulfonamides at 37°, data that should be useful in calculating CA inhibition in physiological and clinical studies. These are difficult to obtain directly due to the very high rate of uncatalyzed CO_2 hydration at higher temperature. The data have intrinsic value in connection with studies of the molecular basis of sulfonamide inhibition of catalysis.

Materials and Methods

Enzyme preparation. CA I and II were prepared from outdated whole human blood by an affinity chromatography technique (15). Membrane-bound CA IV was prepared as a microsomal suspension from bovine kidneys and stored in a buffer solution (25 mM triethanolamine sulfate and 1 mM benzamidine, pH 8.5) (16, 17). The specific activity of this preparation was 200 EU/g of pellet, where EU are enzyme units in the 7-ml barbital system (18). The kinetic values of these isoenzymes have been given previously (4).

Source of sulfonamides. Methazolamide, benzolamide, and sulfanilamide were obtained from American Cyanamid Co. (Pearl River, NJ); ethoxzolamide was obtained from Upjohn (Kalamazoo, MI); MK-507 (dorzolamide [Trusopt]) was obtained from Merck Sharp & Dohme Research Laboratories (West Point, PA); and $CF_3SO_2NH_2$ was obtained from Minnesota Mining and Manufacturing (Minneapolis, MN). The structures and ionization profiles of sulfonamides are shown in Fig. 1. Dorzolamide is of particular interest because it is the first topical CA inhibitor to be used in glaucoma.

Determination of equilibrium and rate constants. Enzyme-sulfonamide dissociation constants K_I were obtained at 0°, 15°, and 23° by a buffer indicator method that we have previously described (4) that monitors the rate of acidification of barbital buffer using the CO_2 hydration reaction catalyzed by CA: $CO_2 + H_2O \rightarrow HCO_3^- + H^+$. K_I determination at temperatures of more than 25° becomes unreliable due to the increasing contribution by the uncatalyzed rate of CO_2 hydration to the overall observed rate (catalyzed and uncatalyzed). This stricture applies to any kinetic method that ultimately depends on CO_2 hydration or dehydration because the 37° uncatalyzed rates are approximately 40-fold greater than at 0°. In the present study, low and nearly constant substrate concentration was maintained across the temperature range with the use of an 8% CO_2/air mixture, providing 5.6 mM CO_2 at 0°, and a 16% CO_2/air mixture at 15° and 23°, providing 7.2 mM and 6.0 mM CO_2, respectively. Sulfonamides and enzyme (two units that increase the uncatalyzed rate by 3-fold) were incubated for 4 min in 5 ml indicator solution at assay temperature in the absence of substrate to ensure equilibrium. The solution was then saturated with the gas mixture, and the reaction was initiated by by addition of 2 ml barbital buffer (50 mM, pH 7.9 = pK at 25°). The decrease in pH is monitored by change in indicator color or by pH electrode. The change in pH is minimal (≤0.5 unit), ensuring that alterations in the ionization state of the sulfonamide- and zinc-ligated water molecule in the enzyme active site are relatively small. The change in buffer pK_a (0.5-unit decrease from 37° to 0° for barbital) and sulfonamide pK_a (average of 0.3-unit decrease in this range) has no effect on data because it has been shown that K_I is invariant between pH 7 and 9 (10).

I_{50} values reflect the concentration of drug that reduces enzyme activity to 1 unit and were obtained by replications in triplicate at several inhibitor concentrations. Inhibition is noncompetitive with respect to substrate CO_2 (11). K_I values were obtained with the following relation, which was derived from classic equilibria (19): $K_I = I_{50} - 1/2\ E_o$, where E_o, the molar enzyme concentration, was calculated from 1 EU = 0.7×10^{-9} M in the 7-ml assay for CA II and 4.9×10^{-9} M in the assay for CA I (20). For CA IV, 1 unit = 1.2×10^{-9} M. K_I was related to the equilibrium constant K_{eq} by the following relation: $K_{eq} = 1/K_I$. K_I values for MK-507 were limited because this compound, like its precursor MK-927, does not inhibit CA I (21). Also, it fails to equilibrate fully with CA II when incubated at 0°, which is unlike the other aromatic and heteroaromatic sulfonamides. It was therefore equilibrated at 25° before assay at 0°.

Association and dissociation rate constants (k_{on} and k_{off}) were determined at 0° and 23° for sulfonamide inhibition of CA II by measuring the rate of approach to equilibrium (22). The rates for sulfonamides with CA I and IV were too rapid to permit use of this method, as were the reactions of $CF_3SO_2NH_2$ and sulfanilamide with CA II (see Results). The procedure is qualitatively similar to that described for K_I except that enzyme and inhibitor are mixed at zero time and then reacted and sampled at time intervals until equilibrium is reached, usually at 4 min. The samples are run in the standard hydration reaction as described. The process yields increasing values for fractional inhibition (i), which becomes constant in the fully equilibrated sample. Values for i, E_o, and I_o (initial enzyme and drug concentration) and seconds of incubation were then used to compute k_{on} and k_{off} (22).

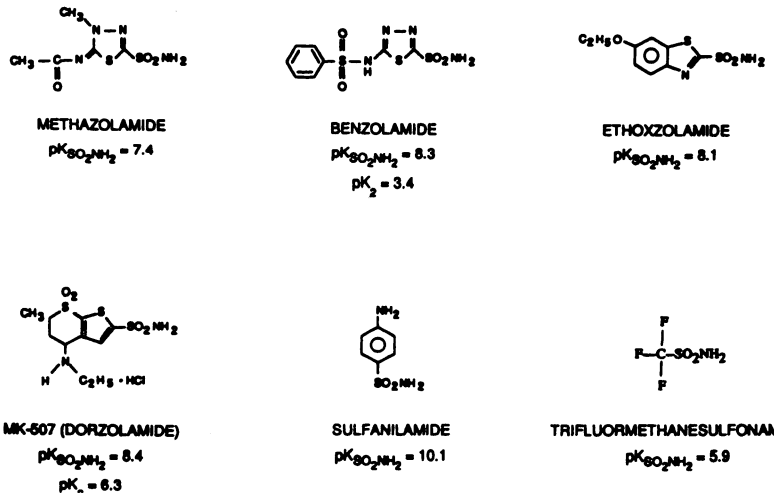

Fig. 1. Structures and ionization profiles of sulfonamides.

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Results

The variation of K_I with temperature for a series of sulfonamides of diverse physicochemical type binding to three CA isoenzymes (I, II, and IV) is shown in Tables 1–3. As determined by the K_I value at a particular temperature, the inhibitory potency of a particular sulfonamide decreases for inhibition of CA isoenzymes in the order II ≥ I > IV, as has been noted previously (4). For all sulfonamide-CA reactions that we studied, the K_I increased with temperature, which is consistent with a negative enthalpy of ligand binding. Despite differences in the magnitude of K_I for sulfonamides and the different isoenzymes, the extent of increase in K_I (0–23°) appeared to be similar for all isoenzymes. Sulfanilamide (2.8–3-fold) and ethoxzolamide (3.2–3.7-fold) produced the smallest increase, whereas $CF_3SO_2NH_2$ (5-fold) produced the largest increase. The mode of acetazolamide binding to CA I and CA II has been shown to be nearly identical (reviewed in Ref. 23). Comparable crystal structure data for sulfonamides and CA IV are not available.

The variation of K_I with temperature was used to obtain estimates for K_I values at 37° (Tables 1–3; from Arrhenius plots of $-\ln K_{eq}$ versus 1/T, where T is the temperature in degrees Kelvin), and these should be useful in physiological studies. The range of K_I increases (in general 6–14-fold) seen over the 0–37° range may represent the range for other sulfonamides binding to CA isoenzymes, as a wide variety of structures were investigated. Determination of sulfonamide K_I at 37° for the hydration reaction is difficult because the rate of uncatalyzed CO_2 hydration becomes greater than the enzymatic rate unless relatively large amounts of enzyme are used. Because $K_I = I_{50} - 1/2\ E_o$, the correction for enzyme concentration then introduces large errors into the estimation of K_I.

Enthalpy values of sulfonamide binding were calculated from the slope of Arrhenius curves (ΔH = slope · R) (Table 4). All ΔH values were in the range of −7 to −12 kcal/mol. The enthalpy values shown for CA II can be compared with the range of −10.8 to −13.8 kcal/mol obtained calorimetrically for the binding of sulfanilamide, benzolamide, and methazolamide to high activity bovine CA and human CA II (12). The apparent enthalpy values of sulfonamide binding reported in Table 4 pertain to equilibrium data obtained at a mean pH of 7.5 in the hydration assay. The enthalpy of $CF_3SO_2NH_2$ binding, approximately −11.6 kcal/mol, was no different from that of sulfonamides such as methazolamide and benzolamide, even though the latter possess heteroaromatic ring structures that have been shown to increase the rate of sulfonamide association and lead to a lowering of K_I (5). This consideration does not apply to $CF_3SO_2NH_2$. Here, the very low molecular mass of the inhibitor apparently results in very high association rates (see later) and consequent lowering of the K_I. This shows that the predominant contribution to the overall enthalpy change associated with sulfonamide binding is that of Zn-$^-$NHSO$_2$R bond formation and not the hydrophobic interaction of the sulfonamide with the active site.

TABLE 1

Effect of temperature on the CA II sulfonamide equilibrium dissociation constant

	K_I			
	0°	15°	23°	37°[a]
			nM	
Methazolamide	4.9 ± 1.2	14.1 ± 2.3	22.9 ± 4.6	54.3 ± 13.3
Benzolamide	0.9 ± 0.2	2.5 ± 0.4	4.2 ± 1.3	9.5 ± 2.4
Ethoxzolamide	0.6 ± 0.1	1.2 ± 0.3	1.9 ± 0.3	3.4 ± 0.8
MK-507	0.8 ± 0.2	2.0 ± 0.3	3.3 ± 0.3	7.4 ± 1.9
$CF_3SO_2NH_2$	3.5 ± 0.9	9.7 ± 1.3	18.5 ± 2.8	43.0 ± 11.7
			μM	
Sulfanilamide	5.2 ± 0.9	11.4 ± 2.2	14.9 ± 3.8	28.1 ± 6.0

[a] Calculated by extrapolation of the Arrhenius plot (see text).
n = 4–9. Values are given as mean ± standard error.

TABLE 2

Effect of temperature on the CA I sulfonamide equilibrium dissociation constant

	K_I			
	0°	15°	23°	37°[a]
			nM	
Methazolamide	8.3 ± 1.6	24.4 ± 5.8	36.8 ± 7.3	92.3 ± 15.0
Benzolamide	2.0 ± 0.4	5.1 ± 0.9	8.5 ± 2.0	17.9 ± 4.2
Ethoxzolamide	1.3 ± 0.3	3.0 ± 0.6	4.7 ± 0.9	9.0 ± 1.6
MK-507	>5 × 10^6	>5 × 10^6	>5 × 10^6	···
$CF_3SO_2NH_2$	4.2 ± 1.1	13.1 ± 3.4	21.4 ± 4.8	54.3 ± 13.7
			μM	
Sulfanilamide	22.5 ± 9.7	46.1 ± 12.0	63.5 ± 15.8	89.7 ± 21.7

[a] Calculated by extrapolation of the Arrhenius plot (see text).
n = 4–9. Except for MK-507, values are given as mean ± standard error.

TABLE 3

Effect of temperature on CA IV sulfonamide equilibrium dissociation constant

	K_I			
	0°	15°	23°	37°[a]
			nM	
Methazolamide	105 ± 20	290 ± 60	530 ± 125	1200 ± 230
Benzolamide	23.8 ± 4.4	57.6 ± 9.8	88.4 ± 17.9	220 ± 35
Ethoxzolamide	15.3 ± 3.6	38.7 ± 8.3	56.9 ± 12.2	115 ± 27
MK-507	31.1 ± 1.4	100 ± 17	160 ± 35	395 ± 85
$CF_3SO_2NH_2$	10.2 ± 1.8	35.0 ± 6.7	59.3 ± 12.5	140 ± 25
			μM	
Sulfanilamide	24.9 ± 4.1	528 ± 7.4	73.4 ± 7.8	144 ± 42

[a] Calculated by extrapolation of the Arrhenius plot (see text).
n = 4–9. Values are given as mean ± standard error.

TABLE 4

Enthalpy of sulfonamide binding to CA isoenzymes

	ΔH[a]		
	CA II	CA I	CA IV
		kcal/mol	
Methazolamide	−10.7 ± 0.2	−10.3 ± 0.2	−9.2 ± 0.2
Benzolamide	−11.0 ± 0.4	−9.2 ± 0.7	−10.9 ± 0.2
Ethoxzolamide	−8.6 ± 0.3	−8.9 ± 0.3	−9.2 ± 0.3
MK-507	−10.3 ± 0.4	···	−11.2 ± 0.3
$CF_3SO_2NH_2$	−11.4 ± 0.2	−11.5 ± 0.3	−12.3 ± 0.2
Sulfanilamide	−7.7 ± 0.2	−7.3 ± 0.2	−7.6 ± 0.2

[a] Obtained from $-\ln K_{eq}$ versus 1/T where slope = ΔH/R, R = 1.98 cal/mol/degree.
Based on 4–9 determinations at each of three temperatures. Values are given as mean ± standard error.

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TABLE 5

Free energy and entropy changes associated with sulfonamide binding to CA isoenzymes at 23°

	CA II		CA I		CA IV	
	$\Delta G°$[a]	ΔS[b]	$\Delta G°$[a]	ΔS[b]	$\Delta G°$[a]	ΔS[b]
	kcal/mol	*eu*	*kcal/mol*	*eu*	*kcal/mol*	*eu*
Methazolamide	−10.3 ± 0.1	−0.9 ± 0.1	−10.0 ± 0.1	−0.9 ± 0.1	−8.5 ± 0.2	−2.5 ± 0.2
Benzolamide	−11.3 ± 0.2	+0.9 ± 0.1	−10.9 ± 0.2	+5.8 ± 0.8	−9.5 ± 0.1	−4.7 ± 0.2
Ethoxzolamide	−11.8 ± 0.1	+10.6 ± 1.0	−11.2 ± 0.1	+7.6 ± 0.7	−9.8 ± 0.1	+2.1 ± 0.2
MK-507	−11.4 ± 0.1	+4.0 ± 0.5	...	...	−9.2 ± 0.1	−6.8 ± 0.5
$CF_3SO_2NH_2$	−10.4 ± 0.1	−3.3 ± 0.2	−10.4 ± 0.2	−3.9 ± 0.5	−9.8 ± 0.2	−8.7 ± 0.4
Sulfanilamide	−6.5 ± 0.1	−4.1 ± 0.3	−5.6 ± 0.2	−5.5 ± 0.5	−5.5 ± 0.1	−7.1 ± 0.6

[a] $\Delta G° = -RT \ln K_{eq}$, where $K_{eq} = 1/K_I$.
[b] $\Delta S = (\Delta H - \Delta G°)/T$; ΔS units, cal/mol/degree (eu).
Values are given as mean ± standard error.

The standard free energies and entropies of sulfonamide binding are given in Table 5. $\Delta G°$ values varied from −5.7 kcal/mol for sulfanilamide binding to CA I to −11.8 kcal/mol for the ethoxzolamide binding to CA II. Associated entropy changes, except for ethoxzolamide, were modest and showed no consistent pattern, again reinforcing the general idea of enthalpy-driven reactions opposed by little or no entropy change. However, for binding of the highly hydrophobic inhibitor ethoxzolamide to CA I and II, this was not the case. Observed entropy changes of +7.8 and +13 EU are significantly larger than for the binding of other sulfonamides and probably reflect the exclusion of bound or structured water from both the enzyme active site and the sulfonamide on binding.

Relatively negative entropy changes accompany the sulfonamide binding reactions of CA IV. The question naturally arises as to whether this may be caused by steric restriction of the approach of sulfonamide and enzyme active site due to anchoring of CA IV to the membrane surface. This was tested by determining the K_I for ethoxzolamide at 0° after CA IV had been liberated from the membrane by pretreatment with 0.5% sodium dodecyl sulfate. The K_I was found not to change from that reported in Table 3, suggesting that the lesser degree of sulfonamide inhibition of CA IV and associated negative entropies are intrinsic properties of this isoenzyme.

The effect of temperature on the association and dissociation rate constants (k_{on} and k_{off}) is given in Table 6 for methazolamide, benzolamide, ethoxzolamide, and CA II. Analogous kinetic constants for the binding of sulfonamides to CA I at 0° and 23° and to CA IV at 23° could not be obtained by use of the approach to equilibrium technique due to the approximately 5-fold faster rates of sulfonamide association with these isoenzymes. This was also true for $CF_3SO_2NH_2$ binding to CA II and IV, which shows an almost instantaneous equilibration with enzyme. However, the inability to measure k_{on} for $CF_3SO_2NH_2$ binding to CA II at 0° despite a K_I similar to that for methazolamide can only mean that k_{off} for this compound is somewhat greater (10-fold) than for most of the other compounds, perhaps 0.1 sec^{-1}, and thus k_{on} is in the range of 10^8 M/sec, which is too fast to measure. For methazolamide and ethoxzolamide, k_{on} and k_{off} values are in good agreement with those previously reported (4, 22). The increase in k_{on} with temperature (0–37°) was 0–2.6-fold for binding of the three sulfonamides to CA II but was 5.4–27-fold for k_{off}. Because the inhibitory potency of sulfonamides (K_I) is determined by the relations of inhibitor association to dissociation, an increase in the magnitude of the latter with temperature appears to be the primary reason for K_I increasing with temperature.

Table 7 shows the apparent activation parameters obtained by analysis of the 0° and 23° kinetic data. These results may be compared with those reported for *p*-nitrobenzene sulfonamide using a fluorescent quenching technique (24). Table 7 shows a fair correlation for methazolamide and benzolamide. For ethoxzolamide, there is good correlation only for $\Delta G‡$. The activation enthalpy values that we report for methazolamide and benzolamide association (3.1–3.8 kcal/mol) are approximately 3 kcal/mol lower than *p*-nitrobenzenesulfonamide association but are within the range of 2.5–4 kcal/mol for diffusion in water, suggesting that for these two sulfonamides association is a diffusion-controlled process (24). For methazolamide and benzolamide, negative entropy changes for association are balanced by equal changes for dissociation, leading to little net entropy change. For ethoxzolamide dissociation, there is a large net negative entropy change, probably corresponding to the reformation of structured water in the active site and on the inhibitor, leading to a large overall positive entropy change for the binding process.

TABLE 6

Variation with temperature of k_{on} and k_{off} for sulfonamide binding to CA II

	k_{on}			k_{off}		
	0°	23°	37°[a]	0°	23°	37°[a]
	M/sec × 10^{-7}			sec^{-1}		
Methazolamide	0.14	0.27	0.37	0.007	0.062	0.192
Benzolamide	0.78	1.3	1.8	0.007	0.055	0.154
Ethoxzolamide	2.9	2.25	2.0	0.016	0.049	0.087

Based on six points for each drug and temperature. See Ref. 22 for calculations. Standard error was 6–23% of the mean values.
[a] 37° values estimated from ln k_{on} or ln k_{off} versus 1/T.

Discussion

The major finding of the present study is the extent to which K_I, k_{on}, and k_{off} increase with temperature. For the binding to CA isoenzymes of a number of highly active sulfonamides (K_I in nM values) of diverse physicochemical type, the extent of this increase from 0° to 37° was in the range of 5.7–14-fold (average, 10.2 ± 0.6-fold). Due to the high catalytic efficiency of certain CA isoenzymes (e.g., II and IV), it is necessary to achieve a high degree of inhibition to elicit physiological responses such as reduced fluid production.

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TABLE 7
Apparent activation parameters (23°) of k_{on} and k_{off} for sulfonamide binding to CA II

	ΔH‡[a]		ΔS‡[b]		ΔG°‡[c]	
	k_{on}	k_{off}	k_{on}	k_{off}	k_{on}	k_{off}
	kcal/mol		eu [cal/(mol·K)]		kcal/mol	
Methazolamide	3.8 ± 0.6	14.5 ± 2.1	−16.2 ± 2.2	−14.8 ± 1.9	8.7 ± 0.3	18.9 ± 0.8
Benzolamide	3.1 ± 0.5	13.8 ± 1.9	−15.3 ± 2.8	−17.5 ± 2.6	7.7 ± 0.2	19.0 ± 1.1
Ethoxzolamide	−2.4 ± 0.2	5.6 ± 0.8	−32.7 ± 7.2	−45.7 ± 8.7	7.3 ± 0.2	19.0 ± 0.9
p-Nitrobenzene sulfonamide[d]	6.6	16.1	−7.9	−10.9	9.0	19.3

Values are mean ± standard error. Data are from Table 6.
[a] $\Delta H‡ = E_{act} - RT$. E_{act} obtained from slope of ln K_{eq} versus 1/T.
[b] $\Delta S = R \ln (k/T) - R \ln (k_B/h) + \Delta H/RT$ and k is k_{on} or k_{off}.
[c] $\Delta G°‡ = -RT \ln (k \cdot h)/(k_B \cdot T)$, where k_B is the Boltzman constant and h is Plank's constant.
[d] From Ref. 24.

However, it is now apparent that the type calculation for enzyme inhibition *in vivo* that we introduced many years ago (reviewed in Ref. 18) must be modified for use with the 37° data and for the likelihood that inhibition of CA IV is the critical isoenzyme in fluid secretion. In solving for fractional inhibition (i) = $I_f/(I_f + K_I)$, one may consider that the free drug concentration in secretory tissue is approximately 10 μM (25). Using the 37° K_I for dorzolamide (400 nM), one obtains a fractional inhibition value of 0.96. This value elicits physiological effects *in vivo*, so the previously stated rule of needing $i > 0.999$, which had been based on 0° inhibition of CA II (18), may not hold.

These results provide a basis for understanding changes in K_I with temperature in terms of the separate rates of sulfonamide association and dissociation. Rate data obtained for methazolamide and benzolamide binding to CA II reveal that increases in k_{off} with temperature (8.4-fold, 0–23°) predominate over those in k_{on} (1.8-fold). For the highly lipophilic and active inhibitor ethoxzolamide, the increase in K_I over the 0–23° range was at least 3.2-fold, attributable entirely to an increase in k_{off}, as k_{on} was unchanged. In general, then, the increase in K_I with temperature is in large part, if not exclusively, due to an increased rate of sulfonamide dissociation.

The inhibitory potency of CA inhibitors may be viewed as a function of two unrelated physicochemical properties. One is the acidity of the RSO_2NH_2 group. Within an homologous series such as the haloalkyl sulfonamides, where there is no hydrophobic effect, K_I is solely a function of sulfonamide pK_a, a relationship anticipated early in CA inhibitor research but only lately demonstrated (14). Strong electron withdrawal (as in $CF_3SO_2NH_2$) leads to stabilization of the sulfonamido anion, resulting in profound reduction of pK_a from 10.4 in methanesulfonamide to 5.9, giving rise to a tightly bound inhibitor. Presumably, this may also result in a lowering of k_{off}, but this remains to be confirmed for haloalkyl sulfonamide binding by use of fast equilibration techniques. The second factor is the hydrophobic interaction of the sulfonamide with the active site of CA, which is lined with hydrophobic amino acids. Heteroaromatic (fused ring and multiring) sulfonamides, which are hydrophobic (as determined by partition into organic solvents), exhibit high rates of association with the active site of CA and consequently have a low K_I (22). We have also shown that the wide range of sulfonamide K_I values (some 5×10^6-fold from methanesulfonamide to MK-507) is in large part a function of the variation in k_{on} (22). Within such a series, pK_a is not an issue because once bound in the active site, the nucleophilic environment of the enzyme-ligated zinc atom forces ionization of the sulfonamide regardless of the alkalinity of the pK_a. A good example is ethoxzolamide, which is perhaps the most inhibitory of all known sulfonamides (K_I = 0.6 nM at 0°, Table 1) but has a SO_2NH_2 pK_a of 8.1. It is of importance that within a homologous series of benzene sulfonamides of identical (high) pK_a values but differing hydrophilicity, inhibitory activity was a function of lipid partition (5). An extreme example is the binding of imidazole (pK_a > 14) as anion to the active site of CA I (13). Some heteroaromatic sulfonamides (e.g., benzolamide) that have show high association rates and low K_I values and, because of a non-SO_2NH_2 ionization, do not partition into organic solvent may nevertheless interact hydrophobically with CA.

These results provide a basis for understanding the degree to which K_I increases with temperature for the sulfonamide binding reactions of CA isoenzymes and yield data for the reactions at body temperature. Additional studies are called for using fast equilibration techniques to clarify the effect of temperature on the rates of sulfonamide association and dissociation to CA I and IV.

References

1. Maren, T. H. The links among biochemistry, physiology and pharmacology in carbonic anhydrase mediated systems, in *Carbonic Anhydrase from Biochemistry and Genetics to Physiology and Clinical Medicine* (F. Botrè, G. Gros, and B. T. Storey, eds.). VCH, New York, 186–207 (1991).
2. Maren, T. H. The kinetics of HCO_3^- synthesis related to fluid secretion, pH control, and CO_2 elimination. *Ann. Rev. Physiol.* **B50:**695–717 (1988).
3. Sanyal, G., E. R. Swenson, N. I. Pessah, and T. H. Maren. The carbon dioxide hydration activity of skeletal muscle carbonic anhydrase: inhibition by sulfonamides and anions. *Mol. Pharmacol.* **22:**211–220 (1982).
4. Maren, T. H., G. C. Wynns, and P. Wistrand. Chemical properties of carbonic anhydrase IV, the membrane-bound enzyme. *Mol. Pharmacol.* **44:**901–905 (1993).
5. King, R. W., and A. S. V. Burgen. Kinetic aspects of structure-activity relations: the binding of sulfonamides by carbonic anhydrase. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* **193:**107–125 (1976).
6. Riepe, M. E., and J. H. Wang. Infrared studies on the mechanism of action of carbonic anhydrase. *J. Biol. Chem.* **243:**2779–2787 (1968).
7. Bertini, I., L. Banci, C. Luchinat, and M. Sola. The interaction of inhibitors with carbonic anhydrase, in *Carbonic Anhydrase from Biochemistry and Genetics to Physiology and Clinical Medicine* (F. Botrè, G. Gros and B. T. Storey, eds.). VCH, New York, 86–94 (1991).
8. Khalifah, R. G., J. I. Rogers, and J. Mukheyees. Interaction of the unique competitive inhibitor imidazole and related compounds with the active site metal of carbonic anhydrase: linkage between pH effects on the inhibitor binding affinity and pH effects on the visible spectra of inhibitor complexes with the cobalt-substituted enzyme. *Biochemistry* **26:**7075–7063 (1987).
9. Khalifah, R. G. and D. N. Silverman. Carbonic anhydrase kinetics and molecular function, in *The Carbonic Anhydrases* (S. J. Dodgson, R. E. Tashian, G. Gros, and N. D. Carter, eds.). Plenum Press, New York, 49–70 (1991).
10. Taylor, P. W., R. W. King, and A. S. V. Burgen. Influence of pH on the kinetics of complex formation between aromatic sulfonamides and human carbonic anhydrase. *Biochemistry* **9:**3894–3902 (1970).

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11. Maren, T. H., and C. Wiley. The *in vitro* activity of sulfonamides against red cell carbonic anhydrases: effect of ionic and substrate variation on the hydration reaction. *J. Med. Chem.* **11**:228–232 (1968).
12. Binford J. S., S. Lindskog, and I. Wadso. A calorimetric study of the binding of sulfonamides and cyanate to carbonic anhydrase. *Biochim. Biophys. Acta* **341**:345–356 (1974).
13. Khalifah, R. G., F. Zhang, J. S. Parr, and E. S. Rowe. Thermodynamics of binding of the CO_2-competitive inhibitor imidazole and related compounds to human carbonic anhydrase I: an isothermal titration calorimetry approach to studying weak binding by displacement with strong inhibitors. *Biochemistry* **32**:2058–3066 (1993).
14. Maren, T. H., and C. W. Conroy. A new class of carbonic anhydrase inhibitor. *J. Biol. Chem.* **268**:26233–26239 (1993).
15. Khalifah, R. G., D. J. Strader, S. H. Bryant, and S. M. Gibson. Carbon-13 nuclear magnetic resonance probe of active-site ionizations in human carbonic anhydrase B. *Biochemistry* **16**:2241–2247 (1977).
16. Wistrand, P. J. Properties of membrane-bound carbonic anhydrase. *Ann. N. Y. Acad. Sci.* **429**:195–206 (1984).
17. Wistrand, P. J., and K.-G. Knuuttila. Renal membrane-bound carbonic anhydrase: purification and properties. *Kidney Int.* **35**:851–859 (1989).
18. Maren, T. H. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiol. Rev.* **47**:595–781 (1967).
19. Maren, T. H. The relation between enzyme inhibition and physiological response in the carbonic anhydrase system. *J. Pharmacol. Exp. Ther.* **139**:140–153 (1963).
20. Conroy, C. W., R. H. Buck, and T. H. Maren. The microchemical detection of carbonic anhydrase in corneal epithelia. *Exp. Eye Res.* **55**:637–640 (1992).
21. Maren, T. H., A. Bar-Ilan, C. W. Conroy, and W. F. Brechue. Chemical and pharmacological properties of MK-927, a sulfonamide carbonic anhydrase inhibitor that lowers intraocular pressure by the topical route. *Exp. Eye Res.* **50**:27–36 (1990).
22. Maren, T. H. Direct measurements of rate constants of sulfonamides with carbonic anhydrase. *Mol. Pharmacol.* **41**:419–426 (1992).
23. Lindskog, L. Carbonic anhydrase, in *Advances in Inorganic Biochemistry*, Vol. 4 (G. L. Eichhorn and L. G. Marzilli, eds.). Elsevier Biomedical, New York 115–170 (1982).
24. Taylor, P. W., R. W. King, and A. S. V. Burgen. Kinetics of complex formation between human carbonic anhydrases and aromatic sulfonamides. *Biochemistry* **9**:2638–2645 (1970).
25. Maren, T. H. The development of topical carbonic anhydrase inhibitors. *J. Glaucoma* **4**:49–62 (1995).

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